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K-*ras* mutations and *RASSF1A* promoter methylation in colorectal cancer

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Human cancer is characterized by genetic and epigenetic alterations. In this study we provide evidence for the interruption of Ras signaling in sporadic colorectal cancer (CRC) by either genetic activation of the K-*ras* oncogene or epigenetic silencing of the putative tumor suppressor gene *RASSF1A*. Paraffin embedded tumor tissue samples from 222 sporadic CRC patients were analysed for K-*ras* codon 12 and codon 13 activating mutations and *RASSF1A* promoter hypermethylation. Overall, K-*ras* mutations were observed in 87 of 222 (39%) and *RASSF1A* methylation was observed in 45 of 222 (20%) of CRCs. Mutation of K-*ras* alone was detected in 76 of 222 (34%) CRCs. *RASSF1A* promoter methylation with wild-type K-*ras* was observed in 34 of 222 (15%) CRCs. In 101 of 222 (46%) CRCs neither K-*ras* mutations nor *RASSF1A* methylation was observed and 11 of 222 (5%) CRCs showed both K-*ras* mutations and *RASSF1A* methylation. These data show that the majority of the studied CRCs with K-*ras* mutations lack *RASSF1A* promoter methylation, an event which occurs predominantly in K-*ras* wild-type CRCs ($P=0.023$, Chi-square test).
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The Ras family of small GTPases includes three highly similar p21 proteins: H-, K- and N-*ras*, which are encoded by different genes and which function as molecular switches in cell proliferation, differentiation and apoptosis signal transduction pathways (Campbell *et al.*, 1998; Crespo and Leon, 2000). Ras proteins, which are located at the inner plasma membrane, are activated transiently as response to extracellular signals such as growth factors, cytokines and hormones that stimulate cell surface receptors (Campbell *et al.*, 1998). The biological activity of Ras proteins is mediated by an effector domain, by which these proteins bind to different effector molecules (Campbell *et al.*, 1998; Crespo and Leon, 2000; Shields *et al.*, 2000). The hallmark of Ras function is a transition between an inactive state, in which the proteins are bound to GDPs and an active state in which conversion to GTPs occurs. This transit is governed by two types of regulatory proteins: guanine nucleotide exchange factors which catalyze the GDP/GTP exchange and GTPase-activating proteins which enhance the intrinsic capacity of Ras proteins to hydrolyze GTP into GDP, thereby returning Ras to the inactive state (Crespo and Leon, 2000).

Mutant, activated forms of Ras proteins, which are frequently observed in cancer, have an impaired GTPase activity rendering the protein resistant to inactivation by regulatory GAP proteins (Bos, 1989; Crespo and Leon, 2000). Known activating mutations are mainly found in codons 12 and 13, and to a lesser extent in codons 59, 61 and 63. The frequency of each *ras* oncogene in tumors varies, depending on the tissue of origin for the neoplasia. H-*ras* mutations are preferentially found in cancers of the skin and in squamous head and neck tumors, whereas N-*ras* mutations are common in hematopoietic malignancies. K-*ras* mutations are mainly detected in adenocarcinomas of the lung, pancreatic carcinomas and CRCs (Rodenhuis, 1992).

Recently a new Ras effector homologue was characterized (Dammann *et al.*, 2000) named RASSF1, which is located at chromosome 3p21.3, a region frequently showing allelic loss in many cancers (Kok *et al.*, 1997). The gene for RASSF1 encodes two major transcripts, *RASSF1A* and *RASSF1C*, which are produced by alternative, CpG island containing promoters and alternative mRNA splicing. The C splice variant of *RASSF1* binds Ras in a GTP-dependent manner and mediates the apoptotic effects of oncogenic Ras (Vos *et al.*, 2000). *RASSF1C* expression is reduced in ovarian tumor cell lines (Vos *et al.*, 2000), but not in breast, lung and kidney cancer cell lines (Burbee *et al.*, 2001; Dreijerink *et al.*, 2001). The *RASSF1A* isoform, which shows homology with the Ras effector Nore 1, has a putative ATM phosphorylation site and binds to the DNA repair protein XPA, which suggests it is involved in DNA repair and cell cycle control (Agathanggelou *et al.*, 2001; Dammann *et al.*, 2000).

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Promoter methylation of *RASSF1A* and subsequent reduction of expression has been recently reported in a very high proportion of small cell lung cancer (Dammann *et al*., 2001a) and nasopharyngeal cancer (Lo *et al*., 2001) and to a lesser degree in non small cell lung cancers (Dammann *et al*., 2000), bladder cancer (Lee *et al*., 2001), breast cancer (Agathanggelou *et al*., 2001; Burbee *et al*., 2001; Dammann *et al*., 2001b), ovarian cancer (Agathanggelou *et al*., 2001; Yoon *et al*., 2001), renal cell carcinoma (Dreijerink *et al*., 2001; Morrissey *et al*., 2001; Yoon *et al*., 2001) and gastric cancer (Byun *et al*., 2001). Although not studied simultaneously in the same tumors, the highest incidence of *RASSF1A* methylation is observed in tumor types with infrequent *ras* gene mutations, i.e. small cell lung cancer and nasopharyngeal cancer (Bos, 1989). This is consistent with observations that simultaneously occurring (epi)genetic alterations of genes in the same signaling pathway are seldomly observed in a single tumor. For example, mutations in the regulatory domain of β-catenin and APC gene were observed to be mutually exclusive in CRC, consistent with their similar effects on β-catenin stability and Tcf activation (Sparks *et al*., 1998). Furthermore, simultaneous inactivation of *Rb* and the $p16^{INK4a}$ locus, which negatively regulates the phosphorylation of Rb, is rarely found in any tumor type (Serrano, 1997).

To test whether this association also holds true for the Ras signaling pathway, we examined K-*ras* mutations and *RASSF1A* promoter methylation in sporadic CRC ($n=222$), a cancer type with a relatively high frequency (40%) of K-*ras* mutations (Rodenhuis, 1992). We detected K-*ras* mutations in 87 of 222 (39%) CRCs, a frequency consistent with previous reports on the mutational frequency in primary colon cancer. Examples of K-*ras* sequencing are shown in Figure 1. We did not observe any association of K-*ras* mutation with age at diagnosis, gender, Dukes stage or location of the tumor (Table 1).

From our hypothesis that K-*ras* gene mutations and *RASSF1A* methylation are mutually exclusive, it would be predicted that the frequency of *RASSF1A* methylation in primary CRCs would be lower than the frequencies observed in tumor types which rarely show *ras* gene mutations (Bos, 1989; Rodenhuis, 1992), i.e. primary small cell lung cancer (71–79%) (Agathanggelou *et al*., 2001; Burbee *et al*., 2001; Dammann *et al*., 2001a), breast cancer (49–62%) (Burbee *et al*., 2001; Dammann *et al*., 2001b), renal cell carcinoma (23–91% (Dreijerink *et al*., 2001; Morrissey *et al*., 2001; Yoon *et al*., 2001) nasopharyngeal cancer (67%) (Lo *et al*., 2001), bladder cancer (35–62%) (Lee *et al*., 2001; Maruyama *et al*., 2001) and neuroblastoma (55%) (Astuti *et al*., 2001).

To study *RASSF1A* methylation, MSP primers were located in the 5′ region of this gene, where methylation has been previously observed and tested by analysing the lung cancer cell line A549 as a methylated control (Dammann *et al*., 2000). DNA from normal breast tissue ($n=4$), normal lung tissue ($n=4$) and normal lymphocytes ($n=3$) was also examined for methylation

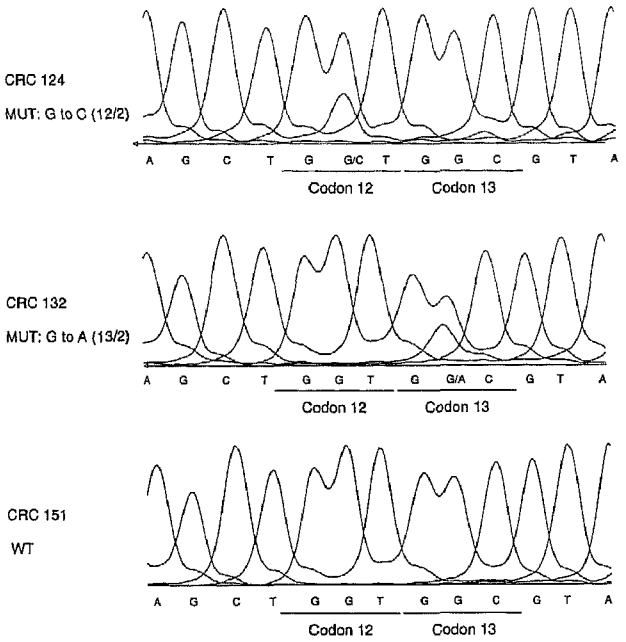

Figure 1 Representative examples of the sequence analysis of codon 12 and 13 of the K-*ras* gene of three CRCs. Mutations were analysed by direct sequencing. First, a flanking PCR product of 179 bp was amplified (annealing temperature 58°C) using the primers 5′-AGG CCT GCT GAA AAT GAC TGA ATA-3′ (sense primer) and 5′-CTG TAT CAA AGA ATG GTC CTG CAC-3′ (antisense primer). The resulting fragment was then used as a template to amplify a 114 bp fragment including codon 12 and codon 13 using the primers 5′-AAA ATG ACT GAA TAT AAA CTT GTG G-3′ (sense primer) and 5′-CTC TAT TGT TGG ATC ATA TTC GTC-3′ (antisense primer). This PCR was performed using one standard and one biotinylated primer, the annealing temperature was 50°C. The PCR product is then captured on a sequencing comb coated with streptavidin (Auto-Load Solid Phase Sequencing Kit, Amersham Pharmacia Biotech), and the non-biotinylated strand is removed by alkaline denaturation. The remaining immobilized strand serves as the template for dideoxy sequencing reactions using a Cy5 labeled primer (5′-CTC TAT TGT TGG ATC ATA TTC GTC CAC-3′) and T7 DNA polymerase (procedure according to manufacturers instructions) and is analysed on the ALFexpress II DNA Analysis System using ALFwin Administration software (Amersham Pharmacia Biotech). CRC 124 shows a G to C mutation (MUT) at the second base of codon 12, CRC 132 shows a G to A mutation at the second base of codon 13 and CRC 151 is wildtype (WT) for K-*ras* codons 12 and 13. The presence of a wildtype sequence in CRCs 124 and 132 is probably the result of the presence of normal, contaminating tissue

changes in this region. The A549 cell line was fully methylated for *RASSF1A*, while DNA from normal lymphocytes, normal breast and normal lung tissue was unmethylated (Figure 2).

In CRC, *RASSF1A* methylation was observed in 45 of 222 (20%) of the tumors. Examples of the *RASSF1A* MSP are shown in Figure 2a. We also analysed DNA from normal colon tissue obtained from six non-cancer patients. None of the six tissues showed *RASSF1A* methylation (Figure 2b). In addition to tissue from non-cancer patients, DNA from normal colon epithelium microdissected from seven CRCs was analysed. In six of seven cases, normal colon DNA was

Table 1 Clinico-pathological parameters of K-*ras* wildtype (WT) and mutant (MUT) and *RASSF1A* unmethylated (U) and methylated (M) CRCs and of the total group of CRCs

	K-ras WT (n=135)	*K-ras* MUT (n=87)	*RASSF1A* U (n=177)	*RASSF1A* M (n=45)	*Total* (n=222)
Mean age (y)	67.7	68.3	67.6	69.1*	67.9
Gender					
M	80 (59.3%)	49 (56.3%)	100 (56.5%)	29 (64.4%)	129 (58.1%)
F	55 (40.7%)	38 (43.7%)	77 (43.5%)	16 (35.6%)	93 (41.9%)
Dukes stage					
A	26 (19.3%)	25 (28.7%)	41 (23.3%)	10 (22.2%)	51 (23.0%)
B	51 (37.8%)	28 (32.2%)	62 (35.0%)	17 (37.8%)	79 (35.6%)
C	36 (26.7%)	20 (23.0%)	44 (24.9%)	12 (26.6%)	56 (25.2%)
D	16 (11.9%)	8 (9.2%)	21 (11.9%)	3 (6.7%)	24 (10.8%)
UK	6 (4.4%)	6 (6.9%)	9 (5.0%)	3 (6.7%)	12 (5.4%)
Location					
Colon	95 (70.4%)	54 (62.1%)	117 (66.1%)	32 (71.1%)	149 (67.1%)
Rectosigmoid	15 (11.1%)	11 (12.6%)	19 (10.7%)	7 (15.6%)	26 (11.7%)
Rectum	25 (18.5%)	22 (25.3%)	41 (23.2%)	6 (13.3%)	47 (21.3%)

*$P=0.033$ T-test. Paraffin embedded CRC tissue samples were obtained from 222 patients who participate in The Netherlands Cohort Study on Diet and Cancer. The study design has been described in detail elsewhere (van den Brandt *et al*., 1990). Record linkage covering the period up to the end of 1993 (7.3 years follow-up), and excluding the first two years of follow-up, resulted in 819 incident CRC patients (ICD-O 153.9, 154.0 and 154.1). The distribution of age, gender, Dukes stage and location of tumor of the patients in this study are representative for the 819 eligible CRC patients. The study protocol was approved by the ethics committee of the University Hospital Maastricht. Differences in mean values or in categorical variables between two groups were tested using the *t*-test and Chi-square test respectively. Data were analysed using SPSS software (version 9.0)

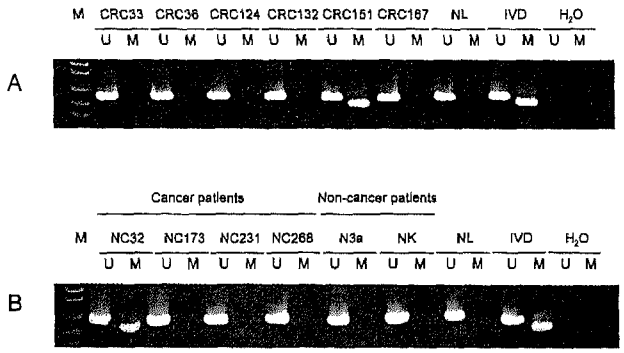

Figure 2 Representative examples of *RASSF1A* MSP reactions of six primary CRCs (a) and of DNA isolated from normal colon epithelium of four CRC patients and two non-cancer patients (b). DNA methylation in the CpG island of *RASSF1A* was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent MSP as described elsewhere (Herman *et al*., 1996). The primers used were 5'-GTT TAG TTT GGA TTT TGG GGG AG-3' (sense primer) and 5'-CCC RCA ACT CAA TAA ACT CAA ACT C-3' (antisense primer). The annealing temperature was 56°C. The resulting 144 bp fragment was used as a template for the MSP-reaction. Primer sequences for the unmethylated reaction were 5'-GGG GTT TGT TTT GTG GTT TTG TTT-3' (sense primer) and 5'-AAC ATA ACC CAA TTA AAC CCA TAC TTC A-3' (antisense primer). Primer sequences for the methylated reaction were 5'-GGG TTC GTT TTG TGG TTT CGT TC-3' (sense primer) and 5'-TAA CCC GAT TAA ACC CGT ACT TCG-3' (antisense primer). All PCRs were performed with controls for unmethylated *RASSF1A* alleles (DNA from normal lymphocytes), methylated *RASSF1A* alleles (normal lymphocyte DNA treated *in vitro* with SssI methyltransferase (New England Biolabs)) and a negative control without DNA. The annealing temperature was 60°C. 10 μl of each PCR reaction was directly loaded onto a nondenaturing 6% polyacrylamide gel, stained with ethidium bromide and visualized under UV illumination. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated *RASSF1A* alleles; the presence of product in those lanes marked M indicates the presence of methylated alleles. All CRCs include amplification with the U primer set, probably a result of the presence of normal, contaminating tissue. Normal lymphocytes (NL) and *in vitro* methylated DNA (IVD) were used as negative and positive controls for *RASSF1A* promoter methylation, respectively. The H_2O control was included in the flanking PCR and subsequently in the *RASSF1A* MSP

unmethylated for *RASSF1A*. In one case, DNA isolated from normal colon tissue showed *RASSF1A* methylation. This patient also had *RASSF1A* methylation in DNA obtained from adjacent tumor tissue (Figure 2b). *RASSF1A* methylation in this 'normal' colon tissue might be explained by the presence of contaminating tumor cells or the presence of a field defect in colon mucosa.

As observed for K-*ras* mutations, *RASSF1A* methylation was not associated with gender, Dukes stage and location of the tumor (Table 1). Age at diagnosis was slightly higher ($P=0.033$, T test) in *RASSF1A* methylated CRCs. *RASSF1A* methylation, however, was largely confined to tumors without K-*ras* mutations. *RASSF1A* promoter methylation without K-*ras* mutations was observed in 34 of 222 (15%) CRCs, while 76 of 222 (34%) CRCs showed K-*ras* gene mutations without *RASSF1A* promoter methylation. This difference between methylation of *RASSF1A* versus K-*ras* mutations was statistically significant ($P=0.023$, two-tailed Chi square test).

Since the majority of mutations in K-*ras* occur in codons 12 and 13, inclusion of codons 59, 61 and 63 in the K-*ras* gene analysis would not be expected to increase the prevalence of K-*ras* mutations in this study significantly. Therefore, our observation that 46% (101 of 222) of the CRCs contain neither K-*ras* codon 12 and 13 mutations nor *RASSF1A* methylation, suggest that other genes in the Ras signal transduction pathway may be altered in CRC. Alternatively, Ras signaling may not be affected in all CRCs.

A small proportion (11/222 (5.0%)) of CRCs had both *RASSF1A* methylation and K-*ras* mutations. These CRCs did not show specific characteristics concerning type of mutations of clinico-pathological parameters. Redundancy in alterations of members of the same pathway is sometimes observed, i.e. $p14^{ARF}$ methylation and *p53* mutations (Esteller *et al*., 2000). One explanation is that the coexistance of alterations

of both *RASSF1A* and *K-ras* is a rare, stochastic event. Alternatively, this could suggest that methylation of *RASSF1A* and mutational activation of *K-ras* are not functionally equivalent.

While the frequency of *RASSF1A* methylation has been reported for many different types of cancer, little data concerning the function of this gene has been published. Exogenous expression of *RASSF1A* cell lines lacking expression decreased *in vitro* colony formation and *in vivo* tumorigenicity (Burbee *et al*., 2001; Dammann *et al*., 2000). No studies have examined whether *RASSF1A* inactivation leads to constitutive activation of the Ras signaling pathway. However, our data provide further evidence for a role in cancer in that inactivation of *RASSF1A* occurs predominantly in CRCs without alteration of K-*ras* itself, and may provide an alternative pathway for affecting Ras signaling. The factors responsible for whether a cell will exhibit DNA methylation changes, gene mutations or allelic losses need to be clarified, although different exposures to environmental carcinogens might contribute to these differences.

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